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A Simple and Sensitive Assay for Determination of Human Anti-Idiotypic Anti-B72.3 Antibodies, which Is not Affected by the Presence of Tumour-Associated Glycoprotein 72

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Summary: An immunoradiometric assay is described for the determination of human anti-idiotypic anti-B72.3 IgG. The latter is formed in ovarian cancer patients after treatment with the murine monoclonal antibody B72.3, which is directed against the tumour-associated glycoprotein 72 (TAG-72). A gel coupled with Fc-specific anti-human IgG antibodies is used as a solid phase for the extraction of serum IgG. The anti-B72.3 IgG is then specifically detected by incubation with radiolabelled B72.3 detector antibodies. Calibration standards were prepared from serum obtained from a patient repeatedly treated with B72.3 antibodies. The concentration of anti-idiotypic anti-B72.3 antibodies was expressed as TAG-72-like arb.units/l. The assay performed with two 60-minute incubation steps is characterized by a high sensitivity (detection limit: 3×10^3 arb.units/l) and precision (coefficients of variation: intra-assay = 6.4% and 5.8% at 80×10^3 arb. units/l and 217×10^3 arb.units/l, inter-assay = 8.7% and 7.1% at 91×10^3 arb.units/l and 212×10^3 arb.units/l) and a good linearity of dilution (recovery after dilution between 99% and 107%). The assay is more specific than previously described methods; no interference was observed by TAG-72 up to 3.3×10^7 arb.units/l. Also, non-specific human anti-mouse antibodies did not cross-react up to 34.8 mg/l. The test may be modified for detection of anti-idiotypic antibodies, which are formed after treatment with other monoclonal antibodies.

Introduction

A novel therapeutic approach to the treatment of malignant neoplasms is the application of unconjugated monoclonal antibodies directed against a tumour-associated antigen expressed on the tumour cells. An indirect mechanism is thought to be involved in the beneficial effect observed in cancer patients after treatment with monoclonal antibodies (1). According to the idiotypic network hypothesis proposed by *Jerne* (2), injection of a monoclonal antibody specific for a tumour-associated antigen induces the formation of anti-idiotypic antibodies which subsequently may induce the formation of anti-anti-idiotypic antibodies, which in return recognize the same epitope on the tumour-associated antigen as on the original antibody. These anti-anti-idiotypic antibodies as well as anti-idiotype reactive T-cells (3) may kill the tumour-cells bearing the tumour-associated antigen by a direct mechanism. Induction of such a humoral idiotypic cascade has been shown after the in vivo application of various monoclonal antibodies (4–7), and the formation of anti-idiotypic antibodies has been favorably correlated with the response of the patients (1, 6, 8, 9).

In most studies, anti-idiotypic antibodies have been measured with an "indirect" assay, in which the anti-idiotypic antibodies are captured by the immobilized original antibody, then detected by incubation with labelled anti-human immunoglobulin G (IgG) or immunoglobulin M antibodies (4, 6, 10). Alternatively, in the direct "sandwich"-assay, the original antibody is used as both capture and detector antibody (9–12). In both types of assay the original antibody bound to a solid phase is incubated with whole serum. Thus, for serum samples containing the original tumour antigen, interference is to be expected, leading to falsely low or falsely high assay results (7, 10, 13, 14).

Recently, in a clinical trial evaluating the effect of repeated infusion of low doses of the murine monoclonal antibody B72.3 in ovarian cancer patients, the formation of anti-idiotypic anti-B72.3 antibodies was shown (9). The B72.3 antibody defines the tumour-associated glycoprotein 72 (TAG-72), a surface antigen expressed in the majority of human epithelial cancers (15, 16), which is also released into the serum (17, 18). Thus, the anti-idiotypic antibody concentration could be assessed only in samples with low TAG-72 concentrations because the TAG-72 present in patient serum cross-reacted with the "sandwich" assay used in this study (19). To allow the determination of human anti-idiotypic anti-B72.3 antibodies, even in the presence of TAG-72, we developed an immunoradio-

metric assay which is not affected by TAG-72. This assay uses a gel coupled with Fc-specific anti-human IgG antibodies to extract the serum IgG, before specific detection of the anti-idiotypic anti-B72.3 antibodies by radiolabelled B72.3 detector antibodies.

Materials and Methods

Patients and serum samples

Serum samples were obtained from ovarian cancer patients (stage III–IV FIGO) who had received one or more infusions of 1 mg of the B72.3 antibody (Oncoscint; Eurocetus, Frankfurt, Germany). Samples drawn before antibody treatment served as control. Additional control samples were obtained from four patients during the in vitro fertilization cycle. Samples with elevated non-specific human anti-mouse antibody concentrations were obtained from four patients treated with radiolabelled $F(ab')_2$ fragments of the murine antibody OC125 (IMACIS-2; Isotopen Diagnostik CIS, Dreieich, Germany) for the purpose of radioimmunodetection (8), and from one patient treated with the murine antibody B43.13 (Biomira, Edmonton, Canada) for the purpose of photodynamic therapy (20). All samples were aliquoted and stored at −20 °C until analysis. The procedures followed in this study were in accordance with the standards of the ethical committee of our faculty.

Coupling of anti-human IgG antibodies to hydrazide gel

Fc-specific anti-human IgG antibodies were coupled to hydrazide gel (Avidchrom Hydrazide Gel F; Sysmex, Hamburg, Germany) according to the procedure described by *Radparvar* et al. (21). For buffer exchange 2 ml of a Fc-specific goat anti-human IgG antibody solution (2.4 g/l; Sigma, Deisenhofen, Germany) were concentrated with a Centricon-30 concentrator (Amicon, Witten, Germany) to an end volume of 0.04 ml and refilled with 2 ml of coupling buffer (0.05 mol/l sodium acetate, pH 5.0). Then 0.2 ml of a sodium *meta*-periodate solution (0.1 mol/l) was added. After incubation for 20 minutes at room temperature, the oxidized antibody sample was applied to a 2 ml hydrazide gel column equilibrated with 16 ml coupling buffer. After a 15-minute incubation at room temperature, the column was washed with 16 ml coupling buffer and 16 ml phosphate-buffered saline (0.01 mol/l sodium phosphate, 0.0027 mol/l potassium chloride, 0.137 mol/l sodium chloride, pH 7.4) to elute the uncoupled antibodies. Then the gel was removed from the column and suspended in phosphate-buffered saline (1 ml gel in 9 ml buffer).

The maximum binding capacity of the gel was determined by incubating 0.1 ml gel with 0.5 ml of human IgG (reagent grade, Sigma, Deisenhofen, Germany; 0.2 g/l) dissolved in assay buffer (1 ml/l Tween-20 in phosphate-buffered saline) for one hour at room temperature with continuous shaking. At the end of the incubation, the gel was sedimented and washed three times with 3 ml assay buffer. The bound human IgG was then eluted by incubation with 0.3 ml glycine buffer (0.1 mol/l glycine-HCl, pH 2.7). After centrifugation the amount of IgG recovered in the supernatant was quantitated by measuring the absorbance at 280 nm.

Standard material

Serum drawn from an ovarian cancer patient two weeks after the fourth B72.3 infusion was used as standard material. A nominal value of 6.1×10^6 arbitrary units/l (arb.units/l) of anti-idiotypic anti-B72.3 antibodies was assigned to this standard material, according to the value measured with the "sandwich assay" described below. Serum drawn from 5 healthy women was pooled and diluted 1/200 with assay buffer to give the zero standard. Dilutions of the standard material were made in zero standard after 1/200 predilution of the standard material with assay buffer to give a range of standards from 0 to 3.05×10^3 arb.units/l. The standards were aliquoted and stored at −20 °C until used.

Immunoradiometric assay (IRMA) for determination of human anti-idiotypic anti-B72.3 IgG

Standard assay protocol: 0.4 ml of the anti-human IgG gel suspension prepared as described above was pipetted into conical polystyrene test tubes and centrifuged at 3500 *g* for 10 minutes. After aspiration of the supernatant, 0.2 ml of standards prepared as described above or 0.2 ml of test serum diluted 1/200 in assay buffer were added and incubated at room temperature for one hour with continuous shaking. At the end of the incubation the gel was sedimented by centrifugation and washed three times with 3 ml assay buffer. Then 0.2 ml of a solution containing ^{125}I-labelled B72.3 detector antibodies (provided as tracer in the B72.3- M-K-S kit; Sorin Biomedica, Düsseldorf, Germany) diluted 2-fold with assay buffer were added to each tube. To block non-specific binding of the B72.3 detector antibodies to human anti-iso/allotypic anti-B72.3 antibodies, the solution was supplemented with non-specific murine IgG: 0.008 ml of murine IgG (reagent grade; Sigma, Deisenhofen, Germany; 20 g/l) were added to 0.232 ml of antibody solution. After incubation for one hour at room temperature with continuous shaking the gel again was sedimented and washed three times with 3 ml assay buffer. The radioactivity bound to the gel was measured.

To evaluate the effect of increasing the amount of gel, in some experiments the test volume in the first incubation step was raised from 0.12 ml (0.1 ml sample + 0.02 ml gel) to 1.2 ml (1.0 ml sample + 0.2 ml gel), followed by a second incubation with 0.5 ml labelled B72.3 detector antibodies.

"Sandwich assay" for determination of anti-idiotypic anti-B72.3 antibodies

For comparison, anti-idiotypic anti-B72.3 antibodies were determined with a homologous "sandwich assay" using the B72.3-M-K-S kit (Sorin Biomedica, Düsseldorf, Germany) with slight modifications. In this commercially available test for TAG-72, anti-B72.3 antibodies can cause a falsely high TAG-72-like assay response. In samples with low real TAG-72 concentrations the results of the B72.3-M-K-S reflect the concentration of the anti-B72.3 antibodies (19). To block anti-iso/allotypic anti-B72.3 antibodies, 0.008 ml of a solution of non-specific murine IgG (20 g/l) was added to 0.232 ml of sample. The concentration of the anti-idiotypic anti-B72.3 antibodies was expressed as TAG-72-like arbitrary units/l.

Determination of real tumour-associated glycoprotein 72

The real TAG-72 concentrations of the serum samples were measured with the ELSA CA 72-4 (Isotopen Diagnostik CIS, Dreieich, Germany) as described elsewhere (19). To eliminate interference by anti-B72.3 antibodies, samples were heated before the TAG-72 determination as described previously, to extract interfering immunoglobulins (19).

Determination of human anti-mouse antibodies

Non-specific human (anti-iso/allotypic) anti-mouse antibodies (HAMAs) were determined with the HAMA-ELISA-medac (Medac, Hamburg, Germany) using mouse IgG as both capture and detector antigen. In patients not treated with murine antibodies the concentration of human anti-mouse antibodies ranged from 0–0.2 mg/l.

Determination of human serum immunoglobulin G

Human serum IgG was determined by radial immunodiffusion on LC-Partigen IgG plates (Behring, Marburg, Germany).

Evaluation of analytical performance

The within-assay precision was calculated from replicate determinations of a sample in a single assay. Between-assay precision was calculated from the values measured for a sample in consecutive

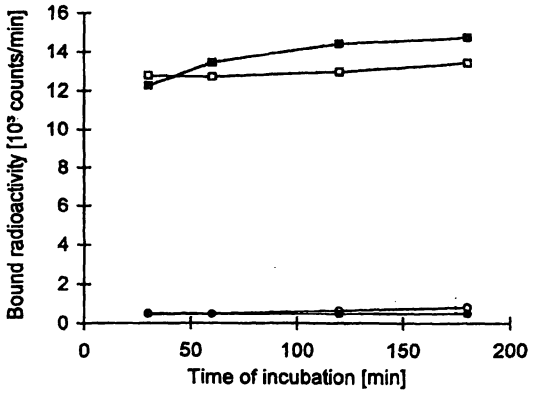

Fig. 1 Variation of incubation time. When the contact time of the zero standard (○) and the 1.53 × 10^3 arb.units/l standard (□) with the solid phase-bound anti-human IgG antibodies was increased from 30 to 180 minutes (first incubation step), this was followed by 60 minutes incubation with the B72.3 detector antibodies. When the contact time with the B72.3 detector antibodies (second incubation step) was increased from 30 to 180 minutes, the zero standard (●) and the 1.53 × 10^3 arb.units/l standard (■) were preincubated with the solid phase bound anti-human IgG antibodies for 60 minutes. Except for variation of the incubation time, the IRMA was carried out according to the standard protocol.

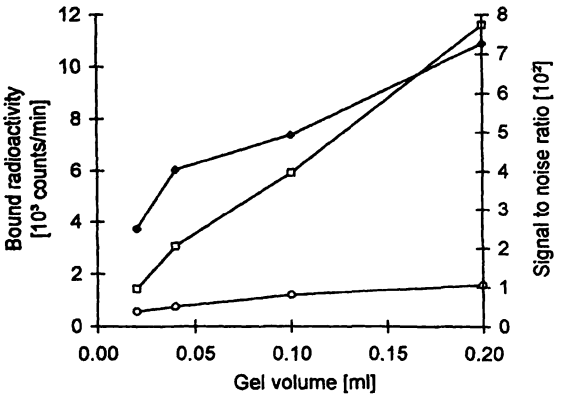

Fig. 2 Variation of gel volume. The signal (□; radioactivity bound in the presence of the 0.76 × 10^3 arb.units/l standard), the noise (○; radioactivity bound in the presence of the zero standard) and the ratio between signal and noise (◆) were determined when the test volume in the first incubation step was raised from 0.12 ml (0.1 ml standard + 0.02 ml gel) to 1.2 ml (1.0 ml standard + 0.2 ml gel), followed by a second incubation with 0.5 ml labelled B72.3 detector antibodies. Except for variation of the incubation volumes the IRMA was carried out according to the standard protocol.

assays. The detection limit was assessed by calculating the concentration which corresponds in the standard curve to the mean signal of 10 replicates of the zero standard + 3 standard deviations. For recovery studies, samples were spiked with a standard pool and the resulting concentration of anti-idiotypic antibodies was measured. For assessment of the linearity of dilution, the anti-idiotypic antibody concentrations measured in samples serially diluted with zero standard (after 1/200 predilution with assay buffer) were compared with the expected values. To examine the effect of elevated concentrations of serum IgG and TAG-72 on assay results, a standard solution with a known concentration of anti-idiotypic antibodies was measured after addition of increasing amounts of human serum IgG and TAG-72, respectively. As a source of TAG-72 we used a mucin prepared from bovine submaxillary glands (Sigma, Deisenhofen, Germany). For comparison of methods, linear regression analysis was performed by the method of *Bablok* et al. (22).

Results

Development of an immunoradiometric assay (IRMA) for determination of human anti-idiotypic anti-B72.3 IgG

As shown in figure 1, for the zero standard as well as the 1.53 × 10^3 arb.units/l standard, only small differences in the resulting test signal were observed when the incubation time of the two incubation steps of the IRMA was increased from 60 to 180 minutes. Thus, for the standard assay protocol we used an incubation time of one hour for each incubation.

The binding capacity for human IgG of two different gel preparations coupled with anti-human IgG antibodies was 37 and 42 µg per 0.04 ml gel. Because of the high IgG concentration in the serum samples (3 to 25 g/l), we diluted the standard material and serum samples 1/200 with assay buffer before applying the standard assay protocol, to guarantee that serum IgG is completely bound to the solid phase. When the volume of gel and sample in the first incubation step was increased (from 0.02 ml gel and 0.1 ml sample up to 0.2 ml gel and 1.0 ml sample) both the signal (bound radioactivity due to the presence of anti-idiotypic antibodies) of the 0.76 × 10^3 arb.units/l standard and the noise (bound radioactivity in the absence of anti-idiotypic antibodies) of the zero standard continued to increase. However, the signal-to-noise ratio also continuously increased (fig. 2). Thus, subsequent assays were performed with 0.04 ml gel and 0.2 ml of standards or serum samples prediluted 1/200 with assay buffer.

We used serum from an ovarian cancer patient treated repeatedly with B72.3 antibodies as standard material. Whereas the heterologous TAG-72 assay detected no real TAG-72 in this standard material, the homologous "sandwich assay" showed apparent TAG-72 value of 6.1 × 10^6 arb.units/l, obviously due to the activity of human anti-idiotypic anti-B72.3 antibodies present in the serum. Thus, an arbitrary anti-idiotypic anti-B72.3 anti-

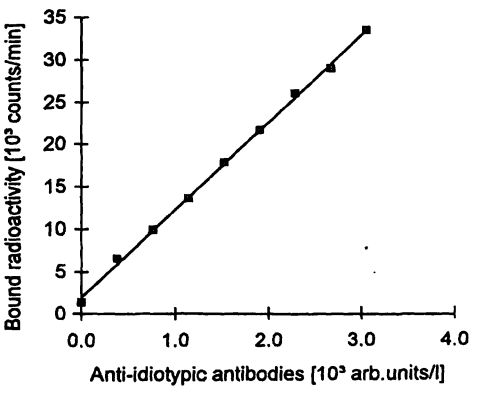

Fig. 3 Dilution curve of standard material. The standard pool was diluted with zero standard after 1/200 predilution with assay buffer. The IRMA was carried out according to the standard protocol.

body concentration of 6.1 × 10^6 TAG-72-like units/l (arb.units/l) was assigned to the standard material. No human anti-mouse antibodies and anti-idiotypic anti-B72.3 antibodies were detected in the serum pool used as zero standard. Standards ranging from 0 to 3.05 × 10^3 arb.units/l were prepared from the standard material by dilution with zero standard. As shown in figure 3 the standard curve measured with the IRMA was linear up to a concentration of 3.05 × 10^3 arb.units/l.

Analytical performance

The minimal detectable concentration assessed on the basis of 10 replicate measurements of the zero calibrator was lower than 0.015 × 10^3 arb.units/l, corresponding to detection limit in the undiluted serum of 3 × 10^3 arb.units/l. Within-assay precision, tested with two samples with mean anti-idiotypic antibody concentrations of 80 and 217 × 10^3 arb.units/l, revealed coefficients of variation of 6.4% and 5.8% (n = 20). The between-assay precision, calculated from values measured in 20 consecutive runs for two samples with mean anti-idiotypic antibody concentrations of 91 and 212 × 10^3 arb.units/l, revealed coefficients of variation of 8.7% and 7.1%. The linearity of dilution was checked for three serum samples with elevated anti-idiotypic antibody concentrations (153, 277, 290 × 10^3 arb.units/l)

Tab. 1 Assay performance of the IRMA standard protocol.
a) Linearity of dilution. Serum samples were serially diluted with zero standard after 1/200 predilution with assay buffer.

Sample identification	Dilution	Anti-idiotypic anti-B72.3 antibodies (10^3 arb.units/l)		Recovery (%)[a]
		Measured	Expected	
10117	1/1	153		
	1/2	76	77	99
	1/4	39	38	102
10618	1/1	277		
	1/2	144	139	104
	1/4	74	69	107
8107	1/1	290		
	1/2	148	145	102
	1/4	76	73	105

b) Recovery. 0.01 ml of the standard pool diluted 1/200 with assay buffer was added to 0.29 ml serum prediluted 1/200 with assay buffer and the resulting anti-idiotypic antibody concentrations were measured.

Sample identification	Anti-idiotypic anti-B72.3 antibodies (10^3 arb.units/l)				Recovery (%)[a]
	Endogenous	Added	Expected	Recovered	
930[b]	14	193	207	208	101
931[b]	4	193	197	192	98
932[b]	<3	193	193	159	82
933[b]	<3	193	193	187	97
600[c]	<3	193	193	188	97
11225[d]	<3	193	193	183	95

c) Interferences by non-specific human anti-mouse antibodies (HAMA). Serum samples with elevated human anti-mouse antibody concentration were measured directly (after 1/200 predilution with assay buffer) or after addition of 0.01 ml of the standard pool (diluted 1/200 with assay buffer) to 0.29 ml of the prediluted serum.

Sample identification	HAMA (mg/l)	Anti-idiotypic anti-B72.3 antibodies (10^3 arb.units/l)				Recovery (%)[a]
		Endogenous	Added	Expected	Recovered	
8027[e]	2.1	<3	–	–	–	–
22788[e]	5.1	8	203	211	219	104
7754[e]	8.3	6	203	209	210	100
6437[e]	34.8	<3	203	203	201	99
11055[f]	12.3	<3	–	–	–	–

[a] Recovery = (anti-idiotypic antibody concentration measured/expected) × 100
[b] Obtained from four in vitro fertilization patients
[c] TAG-72 concentration = 709 × 10^3 arb.units/l
[d] TAG-72 concentration = 6940 × 10^3 arb.units/l
[e] Obtained from four ovarian cancer patients treated with OC125 fragments
[f] Obtained from an ovarian cancer patient treated with B43.13 antibodies

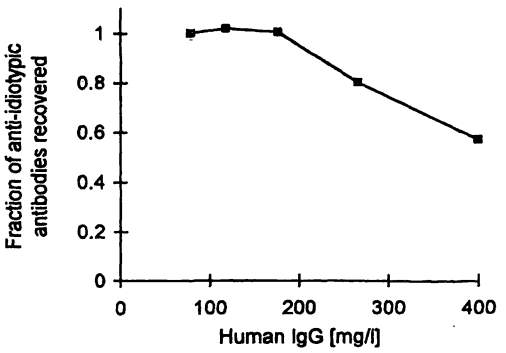

Fig. 4 Recovery of anti-idiotypic anti-B72.3 antibodies in the presence of increasing serum IgG concentration. A 0.76 × 10^3 arb. units/l standard solution prediluted 1/200 with assay buffer was supplemented with increasing amounts of human IgG and measured with the IRMA according to the standard protocol.

obtained from three patients. The concentrations measured after 2- and 4-fold dilutions of the 1/200 prediluted serum samples were only slightly different from the expected values (tab. 1a).

Interferences

As shown in figure 4 the results of the IRMA for a 0.76 × 10^3 arb.units/l standard solution were not affected by addition of increasing amounts of human serum IgG up to an IgG concentration of 175 mg/l (corresponding to a IgG concentration of 35 g/l in the undiluted serum sample). However, in the presence of higher IgG concentrations the assay results were considerably reduced. Furthermore, six serum samples from different patients with serum IgG concentrations from 11 to 18 g/l, showed almost complete recovery of added anti-idiotypic antibodies (tab. 1b), confirming that the different IgG concentrations of the samples have no effect on the assay results.

As shown in table 1b, the two samples with elevated TAG-72 concentrations (sample identification: 600 and 11225) showed almost complete recovery of anti-idiotypic antibodies, indicating that the TAG-72 antigen has no effect upon the results of the IRMA. In the same way, increasing the TAG-72 concentration up to 3.3 × 10^7 arb.units/l, by addition of bovine mucin containing TAG-72-like immunoreactive material, has virtually no effect on the results measured in a 1.15 × 10^3 arb.units/l standard solution (apparent concentration of the standard in the presence of 3.3 × 10^7 arb.units/l TAG-72 = 1.19 × 10^3 arb.units/l).

The apparent anti-idiotypic anti-B72.3 antibody concentration measured in five serum samples from patients treated with the murine antibodies OC125 (four patients) and B43.13 (one patient), with non-specific human anti-mouse antibody concentrations ranging from 2.1 to 34.8 mg/l, did not exceed 8 × 10^3 arb.units/l. Also anti-idiotypic antibodies added to three of the samples were recovered almost completely (tab. 1c).

Clinical evaluation

As shown in table 2, in the samples obtained from 16 ovarian cancer patients before treatment with B72.3 antibodies, the concentration of anti-idiotypic anti-B72.3 antibodies did not exceed 12 × 10^3 arb.units/l. This is in accordance with the results measured in the control samples (tab. 1b). However, after infusion of B72.3 antibodies, 9 patients showed grossly elevated anti-idiotypic antibody concentrations ranging from 204 to 2164 × 10^3 arb.units/l; in 4 patients values were slightly elevated ranging from 27 to 66 × 10^3 arb.units/l; anti-idiotypic antibodies were undetectable in only 3 patients. The human anti-mouse antibodies concentration of the samples drawn after antibody treatment ranged from < 0.1 to 4.2 mg/l.

Figure 5 shows the time course of the concentration of the anti-idiotypic anti-B72.3 antibodies measured with the IRMA and with the "sandwich" assay, respectively, in serum samples drawn from an ovarian cancer patient treated three times with B72.3 antibodies. Three weeks after the third B72.3 infusion, the IRMA detected an increase of the anti-idiotypic antibody concentration up to 587 × 10^3 arb.units/l followed by a decrease to 20 x10^3 arb.units/l five months later. Also the values measured with the "sandwich" assay rose three weeks after the third infusion to 443 × 10^3 arb.units/l followed by an initial decrease to 187 × 10^3 arb.units/l ten weeks later. However, the values then again rose up to 260 × 10^3 arb.units/l, obviously due to the continous increase of the TAG-72 concentration measured in the same period.

Tab. 2 Anti-idiotypic anti-B72.3 antibody concentration in patients treated with the B72.3 antibody. Serum samples drawn from 16 patients before and after B72.3 infusion were measured with the IRMA according to the standard protocol.

Patients	Number of infusions	Anti-idiotypic anti-B72.3 antibodies (10^3 arb.units/l)	
		before treatment	after last infusion
EH	2	4	<3
KE	2	<3	<3
RC	6	<3	<3
WU	3	<3	27
HI	1	<3	34
VM	3	<3	43
MU	3	<3	66
BB	4	<3	204
SR	1	<3	420
AM	3	<3	470
BI	4	<3	526
BM	3	<3	587
FR	3	<3	793
MH	6	12	815
SE	3	<3	1572
TL	1	6	2164

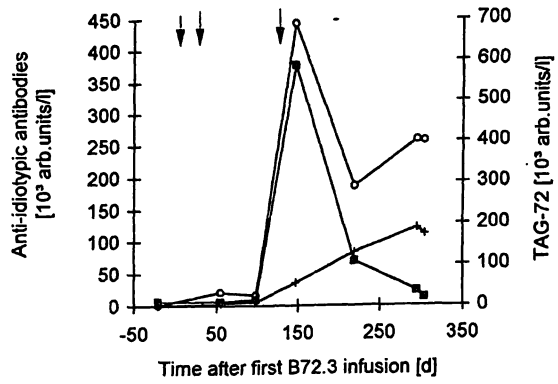

Fig. 5 Typical time course of the formation of anti-idiotypic anti-B72.3 antibodies measured with the IRMA (□) and the "sandwich" assay (○), respectively, during repeated treatment with B72.3 antibodies in an ovarian cancer patient with increasing TAG-72 serum concentration (+). Arrows indicate the time of OC125 infusions.

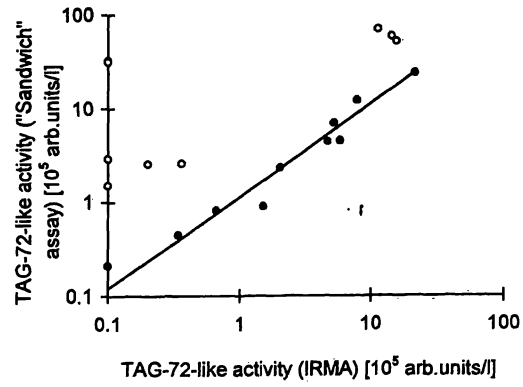

Fig. 6 Comparison of the results measured with the IRMA and the "sandwich" assay, in serum samples obtained from patients after treatment with B72.3 antibodies with low (●) and elevated (○) TAG-72 concentrations. The regression line was calculated for the values measured in the samples with low TAG-72 concentration.

In figure 6 the values measured with the IRMA are compared with the results of the "sandwich" assay. For 10 samples with low TAG-72 concentrations there was a good correlation between both rests ($r = 0.981$; regression line $y = 1.1\,x + 7.6$). For 9 samples with TAG-72 concentrations ranging from 189 to 5490 $\times 10^3$ arb.units/l the values measured with the "sandwich" assay were considerably higher than those from the IRMA.

Discussion

The advantage of the IRMA described here is that the human serum IgG is first selectively extracted by adsorption to solid phase coupled anti-human IgG antibodies. Thus, serum components which may react with the B72.3 detector antibodies can be removed by a washing step and possible interference can be excluded. The data also confirm that very high TAG-72 concentrations have no effect upon the results of the IRMA, whereas the "sandwich" assay gave falsely high values, due to cross-reaction with TAG-72.

In most assays for detection of anti-idiotypic antibodies described in various papers, the murine antibody bearing the original idiotype is coupled to a solid phase and incubated with the serum samples (4, 6, 9–12, 14). Thus, interfering serum components including circulating antigen can react with the immobilized antibody leading to falsely low or falsely high assay results (7, 10, 13, 14). In patients treated with the anti-TAG-72 antibody B72.3 or the anti-CA-125 antibody OC125, which are directed against antigens which can be present in serum in very high concentrations, interference by elevated antigen concentrations in the patient serum can be a problem.

In order to eliminate interference by circulating antigen in the determination of anti-idiotypic anti-B72.3 antibodies, *Ferroni* et al. (14) proposed that the TAG-72 be removed from patient serum by adsorption to the solid phase coupled anti-TAG-72 antibody CC49 which recognizes a separate epitope on the TAG-72 antigen (23). This is a time-consuming procedure and an additional antibody is required which specifically binds the antigen but does not react with the anti-idiotypic antibodies. A more simple method has been described by *Moseley* et al. (13). For determination of human antibodies formed against the anti-CA-125 antibody OC125 they used microtitre wells coated with protein A to extract the immunoglobulins from serum. The anti-idiotypic anti-OC125 antibodies bound to protein A are subsequently detected by incubation with radiolabelled $F(ab)_2$ fragments of the OC125. This may be a simple assay, but $F(ab)_2$ fragments of the original antibody must be available to avoid binding of detector antibodies to protein A on the solid phase. Further problems arise from the high concentration of non-specific IgG present in human serum samples. Because of the limited binding capacity of the protein A on the solid phase (10 µg per well), serum samples must be prediluted 1/100 for correct determination, leading to a decline of sensitivity (13). In the IRMA described here the sensitivity is also limited by the binding capacity of the solid phase for human serum IgG. However, this problem can be compensated partly by increasing the amount of gel used in the first incubation step. On the other hand, the density of antibodies coupled to the hydrazide gel can be substantially enhanced by increasing the amount of antibody reacted (21). This may be a further way of increasing the binding capacity of the solid phase in order to achieve an improved analytical sensitivity.

A further advantage of the IRMA is that, because of the specificity of the capture antibodies used, only anti-idiotypic antibodies of the IgG class are detected. The

assay may be adapted for the determination of anti-idiotypic antibodies of the IgM class by using a gel coupled with anti-human-IgM capture antibodies.

The validity of the IRMA is confirmed by the data obtained for serum samples from patients treated with B72.3 antibodies. In samples drawn before antibody treatment, the IRMA detected no or only marginally elevated anti-idiotypic anti-B72.3 IgG, thereby indicating that the assay is highly specific. Obviously, non-specific interference, as observed with the "indirect" assay format (10), does not affect the IRMA. Also, no false positive values were found in samples with high concentrations of non-specific human anti-mouse antibodies up to 34.8 mg/l. Thus, the amount of murine IgG added to the detector antibodies of the IRMA is sufficient to block the human anti-mouse antibodies activity of the samples drawn after B72.3 treatment which did not exceed 4.2 mg/l.

In 81% of the posttreatment samples we found elevated anti-idiotypic anti-B72.3 IgG concentrations, indicating that the assay is highly sensitive. The time course of the appearance of anti-idiotypic antibodies during antibody treatment shows a good correlation between the time of antibody infusion and the appearance of anti-idiotypic antibodies. The validity of the results is further confirmed by the coincidence with the values measured with the "sandwich" assay for samples with low TAG-72 concentration. However, because the "sandwich" assay detects all classes of anti-idiotypic antibodies, the good agreement between both assays suggests that in the samples compared, the concentration of the anti-idiotypic IgG antibodies is considerably higher than that of the anti-idiotypic antibodies of the other immunoglobulin classes. This may be due to the fact that only one of these samples was drawn within the first weeks following to the first antibody infusion. As reported by *Courtenay-Luck* et al. (4), the immune response to repeated antibody infusions is mainly of the IgG class.

Because the only specific reagent required for the IRMA is the labelled original antibody, the test may be modified to detect anti-idiotypic antibodies, which were formed after treatment with other monoclonal antibodies. At present we have performed preliminary experiments which suggest that this assay design also can be used for determination of human anti-idiotypic antibodies directed against the anti-CA-125 antibodies B43.13 and OC125.

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